AFAMRL-TR-84-059

CELLULAR BIOCHEMISTRY AND CYTOGENETICS IN A RAT LUNG TUMOR MODEL

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OCTOBER 1984

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

BRUCE O. STUART, PhD
Director Toxic Hazards Division
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ECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for Public Release; Distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) AFAMRL-TR-84-059	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Regents of the University of California 6b. OFFICE SYMBOL *(If applicable)*	7a. NAME OF MONITORING ORGANIZATION AFAMRL Toxic Hazards Division
6c. ADDRESS *(City, State and ZIP Code)* University of California, Irvine Irvine, California 92717	7b. ADDRESS *(City, State and ZIP Code)* AMD, AFSC, Wright-Patterson AFB Ohio, 45433
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b. OFFICE SYMBOL *(If applicable)*	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F33615-80-C-0512
8c. ADDRESS *(City, State and ZIP Code)*	10. SOURCE OF FUNDING NOS.

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT NO.
622027	6302	01	15

11. TITLE *(Include Security Classification)*
Cellular Biochemistry and Cytogenetics in a Rat Lung Tumor Model

12. PERSONAL AUTHOR(S)
Ronald E. Rasmussen, Ph.D., Arthur T. Fong.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT *(Yr., Mo., Day)*	15. PAGE COUNT
Annual	FROM 83-7-1 TO 84-6-30	1984, October	23

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS *(Continue on reverse if necessary and identify by block number)*		
FIELD	GROUP	SUB. GR.	Rat	Lung Cancer	Benzo(a)pyrene
			Mouse	Lung Cell Isolation	3-Methylcholanthrene
			Hamster	Asbestos	Diethylnitrosamine

19. ABSTRACT *(Continue on reverse if necessary and identify by block number)*

Fischer 344 rats were treated intratracheally with 3-methylcholanthrene (MCA) and/or asbestos. At a dose of 5 x 1.0 mg given at 2-week intervals, MCA alone induced squamous cell carcinomas of the lung in 33% of treated rats. In vivo incorporation of ^{3}H-thymidine (^{3}H-dThd) into lung cell DNA was not different between treated and control rats at any time after treatment, even when tumor was present. Treatment with asbestos in addition to MCA did not produce an increase in ^{3}H-dThd incorporation into lung cell DNA and did not enhance tumor formation during the 9 months of the experiment.

Lung cells were isolated from rats and mice and separated using centrifugal elutriation to obtain preparations enriched in type II alveolar cells and nonciliated bronchiolar epithelial cells (Clara cells). Studies of metabolism of ^{3}H-benzo(a)pyrene (^{3}H-BaP) by these cell preparations have shown that enzyme activity was induced in both cell types by pretreatment of the animals with inducers of P-450 monooxygenases. In both species Clara cells were much more active in metabolism of ^{3}H-BaP than were type II cells. (Cont.)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☒ DTIC USERS ☐	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE NUMBER *(Include Area Code)*	22c. OFFICE SYMBOL
AFAMRL Monitor: M. K. Pinkerton, AFAMRL/THT	513-255-3364	

DD FORM 1473, 83 APR EDITION OF 1 JAN 73 IS OBSOLETE.

SECURITY CLASSIFICATION OF THIS PAGE

(Block 19, continued)
In the rat, but not in the mouse, the profile of metabolites of ^{3}H-BaP was quantitatively different between the type II and Clara cells, with the type II cells producing relatively more of the monohydroxy derivatives of ^{3}H-BaP. These differences may be related to the susceptibility of the cell types to neoplastic transformation by polycyclic hydrocarbons.

Formation of ethylguanines in DNA of hamsters has been studied following treatment with diethylnitrosamine (DEN) which induces carcinomas in the respiratory tract, mainly in the trachea. Chronic treatment with DEN (20 mg/kg b.w., s.c. twice weekly for 8 weeks) produced accumulation of O^6-ethylguanine (O^6-EtG) in lung and liver, but not kidney DNA. On the other hand, the level of 7-ethylguanine (7-EtG) did not increase beyond that found 24 hr after the first treatment. Pretreatment with DEN (20 mg/kg b.w., s.c. twice weekly for 4 weeks) did not alter the levels of 7-EtG or O^6-EtG found in liver or lung after a challenge dose of DEN (150 mg/kg b.w., i.p.) as compared to saline-pretreated controls.

Incorporation of ^{3}H-dThd into hamster lung and liver DNA was studied by autoradiography following chronic or acute exposure to DEN. During chronic exposure the labeling index (ratio of labeled to unlabeled cells) of tracheal epithelial and liver hepatocytes increased during treatment, reaching 19% for tracheal epithelial cells and 10% for hepatocytes, compared to less than 2% and 1%, respectively, for controls. Bronchiolar epithelial cells and type II alveolar cells showed a slight increase in labeling index. After a single treatment with DEN (150 mg/kg b.w., i.p.) the labeling index for tracheal epithelial cells and hepatocytes reached peak values of 14% and 18%, respectively, 4 days posttreatment. The peak of labeling of hepatocytes coincided with liver necrosis caused by the DEN. These results show that accumulation of O^6-EtG occurs in the target organ for carcinogenesis (lung) and that cytotoxi effects are also produced in this organ. Liver may also be a target for DEN-induced carcinogenesis, but, in experimental tests, the hamsters die of tracheal tumors before liver tumors have time to develop.

PREFACE

This is the fourth annual report of the Cytology Cell Biology and Cytogenetics section of the Toxic Hazards Research program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under contract number F33615-80-C-0512. The report describes research activities at UCI and collaborative studies conducted at the Toxic Hazards Research Unit (THRU) of the AFAMRL, Wright-Patterson AFB, Ohio, during the contract period 1 July 1983 through 30 June 1984. During this period, T.T. Crocker, M.D., was Principal Investigator for the contract, and Ronald E. Rasmussen, Ph.D., directed the studies at UCI. Technical personnel at UCI were Staff Research Associates Marcia Witte and Mary Hawley, and Research Assistant Arthur Fong. At the THRU, Edwin R. Kinkead was Study Director and R. Scott Bowers was Research Associate. Technical Monitor for the Air Force was M. K. Pinkerton, AFAMRL/THT, Wright-Patterson AFB, Ohio.

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INTRODUCTION

Statement of the Problem

Carcinogenesis is usually viewed as a multistep process involving, for example, metabolic activation of carcinogens, binding of carcinogens to DNA or other biomolecules, cell toxicity and stimulation of cell proliferation, and effects on the immune system. The initiation step is considered to occur in a particular cell ("target cell") which has characteristics that make it especially susceptible to the transforming effects of chemical carcinogens. These characteristics are presumed to include 1) the ability to metabolically activate procarcinogens to their ultimate carcinogenic form, and 2) the potential for proliferation. In the respiratory system, there are several cell types that appear to meet these criteria; however, the tumors that appear following treatment with carcinogens seem to arise from only a few cell types. Most respiratory tumors have characteristics of either tracheobronchial epithelial cells (e. g. carcinomas) or type II alveolar cells (adenomas). Other cells such as alveolar macrophages and endothelial cells are known to be metabolically active and to be capable of cell division, but tumors arising from these cells are unusual or unknown.

The central question addressed in the studies reported here is "What is unique about cells in target tissues that become transformed as compared to non-target tissues or cells?". The question has been approached in 3 ways.

First, experiments were designed to examine cellular changes that occur in vivo in the rat after intratracheal treatment with the carcinogen 3-methylcholanthrene (MCA) and/or asbestos. Previous work has established that MCA alone could induce squamous cell carcinomas in the rat lung in a dose-dependent manner. The earliest change seen was a proliferation of epithelial cells in terminal bronchioles. The questions asked in the experiments during the current year were (1) at what point in the process of tumorigenesis could evidence of cell proliferation be detected, either through analysis of tissue sections or by incorporation of radiotracers (^{3}H-thymidine, ^{3}H-dThd) by proliferating cells, and (2) does treatment with asbestos affect cell proliferation in a way that might influence tumorigenesis.

In a second series of experiments, which is still underway, a different animal model has been used to study cytotoxic effects and binding of a carcinogen to DNA in target and non-target organs for carcinogenesis. The Syrian golden hamster is susceptible to carcinogenesis by diethylnitrosamine, (DEN), and develops tracheobronchial tumors without regard to the route of administration. The tumors appear to be of epithelial origin, with the non-ciliated Clara cells being a likely target. During the present year, binding of ethyl groups derived from DEN to guanine residues in lung, liver, and kidney DNA has been examined with respect to dose-response and accumulation of alkyl-guanine during chronic exposure. Also, cell proliferation in target and non-target cell populations has been studied using ^{3}H-dThd labeling techniques. The results to date suggest that cell toxicity and binding of DEN to DNA is greatest in those cell populations containing cells susceptible to carcinogenesis by DEN.

Finally, experiments have been initiated in which specific rat lung cell types are being studied for their ability to metabolize hydrocarbon carcinogens. Dispersed cell suspensions are obtained by enzymatic treatment of lung tissue, and the cells are separated on the basis of size using centrifugal elutriation. The cells of most interest are the non-ciliated epithelial cells and Type II alveolar cells because of the evidence that they are precursors of lung tumors. The separation of these cell types has allowed studies of their metabolism of a known lung carcinogen, benzo(a)pyrene (BaP) to its

carcinogenic form. These studies will be extended to include lung cells from mice and hamsters.

SPECIFIC AIMS

Two projects are underway in parallel in this subprogram. The first uses the rat lung tumor system in which squamous cell carcinomas can be induced by polycyclic aromatic hydrocarbon (PAH) such as MCA or BaP. The second project employs a respiratory carcinogen model in which tumors can be induced in the Syrian hamster respiratory tract by administration of diethylnitrosamine (DEN).

For the rat lung tumor system the specific aims are:

1. To conduct studies of the effect of 3-methylchlanthrene (MCA) on DNA synthesis and cell proliferation in the rat lung.

2. To treat rats intratracheally with MCA and asbestos and measure effects on cell proliferation as this may relate to tumorigenesis.

3. To separate lung cells from rats and measure their metabolism of ^{3}H-BaP in vitro.

For the hamster DEN lung tumor system the specific aims are:

4. In the hamster model system, to measure formation of 7-ethylguanine (7-EtG) and O^6-ethylguanine (O^6-EtG) during acute and chronic treatment with DEN.

5. To study cell proliferation in the hamster lung and liver after acute or chronic exposure to DEN.

MATERIALS AND METHODS

The Rat Lung Tumor Model

Intratracheal inoculation with gel-saline (0.2% gelatin in 0.9% NaCl) suspensions of MCA crystals will produce squamous cell carcinomas (SCC) in the Fischer 344 rat (Schreiber et al., 1972). We have previously reported (Rasmussen, 1983; Rasmussen et al., 1984) that the induction of these tumors is dose-related, and that elevation of PAH-metabolizing enzymes in lung and liver is produced by the MCA treatment. The details of treatment of the rats have been reported (Rasmussen, 1983). Briefly, MCA was recrystallized from benzene, ground in a mortar and pestle, and suspended in gel-saline to form a slurry of which 0.1 mL was injected into the trachea of Halothane- or Metofane-anesthetized rats. For tumor induction, rats were treated 5 times at 2-week intervals. Depending on the dose, some rats had SCC by the end of the treatment phase (5 x 5 mg MCA) while at lower doses, a longer latent period was found, and at 5 x 0.1mg MCA, no tumors were found among 25 rats treated.

Treatment with suspensions of asbestos also was as described previously (Rasmussen, 1983). A sample of asbestos (UICC chrysotile) was shaken in distilled water with glass beads and allowed to settle. The fibers remaining in suspension after 1 hr at room temperature were collected by filtration and resuspended in gel-saline for inoculation of the rats.

Experimental Treatment to Examine Cytologic Changes in Rat Lung During Tumorgenesis

Two experiments were initiated during the previous contract year to examine cytologic changes induced by MCA in the rat lung which may be related to eventual tumor development.

Treatment of rats with MCA and housing for observation of tumor development was conducted at the THRU. Once rats had been entered on the study, the animals and their bedding were considered to be contaminated, and appropriate safety precautions were observed to avoid exposure of personnel to MCA. The first experiment, begun in January 1983, was designed to determine (1) whether treatment with a carcinogen produced cell proliferation in the lung that could be detected as increased incorporation of ^{3}H-dThd in vivo, and (2) whether cell cultures prepared from lungs of MCA-treated rats contained significant numbers of cells capable of proliferation in vitro, as compared to cultures from control rats. The treatment and sacrifice schedule for this experiment is shown in Table 1. The dose selected for this experiment was based on previous work, and was expected to produce SCC in 50% of the treated rats. At the times indicated, groups of rats were sacrificed and the lungs either fixed in situ with 10% buffered formalin or removed aseptically and sent on ice to UCI for culture. When lungs were to be fixed with formalin, the rats were given 200 uCi ^{3}H-dThd by i.p. injection 1 hour before sacrifice. The fixed lungs were then sent to UCI for analysis of radioactivity and preparation of paraffin sections for histologic examination.

Table 1

Treatment and Sacrifice Schedule for Cytologic and Cytogenetic Changes in the Rat Lung During Tumorigenesis

DATE	ACTION	SHELF	VEHICLE	1.0 MG MCA
1-24-83	Treat	25 Rats	50 Rats	50 Rats
2-7-83	Treat	25	50	50
2-21-83	Treat	25	50	50
3-7-83	Treat	25	50	50
3-21-83	Treat	25	50	50
4-4-83	Sacrifice	5	5	5
5-9-83	Sacrifice	5	5	5
6-13-83	Sacrifice	5	5	5
7-18-83	Sacrifice	5	5	5
8-22-83	Sacrifice	5	5	5
9-26-83	Sacrifice	5	5	5
10-31-83	Sacrifice	5	5	5
12-5-83	Sacrifice	5	5	5
1-9-84	Sacrifice	5	5	5
3-13-84	Sacrifice	5	5	5

The second experiment was begun in April 1983. The aims of this experiment were to examine the effects of combined treatment with MCA and asbestos on the cytology and tumor formation in the rat lung

The experimental treatment and sacrifice schedule is given in Table 2. As above, the rats were treated and housed at the THRU prior to sacrifice, at which time the rats were given ^{3}H-dThd, and the lungs fixed as above. Whole formalin-inflated lungs were sent to UCI for analysis.

Table 2

Treatment and Sacrifice Schedule for Promotional Effects of Asbestos on Tumorigenesis by MCA

DATE	ACTION	GEL-SALINE	0.1 MG ASB.	0.25 MG MCA	ASB + MCA
4-6-83	Treat	25 G.S.	25 ASB.	25 G.S.	25 ASB.
4-13-83	Treat	25 G.S.	25 G.S.	25 MCA	25 MCA
4-20-83	Treat	25 G.S.	25 ASB	25 G.S.	25 ASB.
4-27-83	Treat	25 G.S.	25 G.S.	25 MCA	25 MCA
5-4-83	Treat	25 G.S.	25 ASB.	25 G.S.	25 ASB.
5-11-83	Treat	25 G.S.	25 G.S.	25 MCA	25 MCA
5-18-83	Treat	25 G.S.	25 ASB.	25 G.S.	25 ASB.
5-25-83	Treat	25 G.S.	25 G.S.	25 MCA.	25 MCA
6-1-83	Treat	25 G.S.	25 ASB.	25 G.S.	25 ASB.
6-8-83	Treat	25 G.S.	25 G.S.	25 MCA	25 MCA
6-20-83	Sacrif.	2	2	2	2
7-25-83	Sacrif.	5	5	5	5
9-5-83	Sacrif.	5	5	5	5
10-17-83	Sacrif.	5	5	5	5
12-12-83	Sacrif.	5	5	5	5

Analysis of Lung Tissue for Incorporation of ^{3}H-dThd

Small samples of formalin-fixed lung tissue of approximately 10 mg dry weight were passed successively through 70%, 95% and 100%, ethanol, remaining at least 16 hours in each solvent. The tissue pieces were dried under vacuum and weighed to the nearest 0.01 mg. To dissolve the tissue, 100 uL of water and 1.0 mL tissue solubilizer (NCS, Amersham) were added to the tissue samples in 20 ml scintillation vials, and the vials were sealed and incubated overnight at 50^{o}C. This procedure completely dissolved the tissue. The solution was neutralized with glacial acetic acid, 10 mL of scintillation solution (ACS, Amersham) was added, and the samples were counted in a Tracor scintillation spectrometer. Specific radioactivity was calculated on the basis of ^{3}H-dpm per mg dry weight. Since ^{3}H-dThd is incorporated essentially exclusively into cellular DNA, this measurement reflects cellular proliferation in the lung tissue.

Paraffin sections of all fixed lungs were prepared for autoradiography. All lung lobes of formalin-fixed lungs were sampled according to the scheme described by Dungworth et al. (1976). The sections were cut at 4-5 um, dewaxed, and dipped in Kodak NTB-2 emulsion diluted 1:1 with water. After drying, the slides were stored at 4^{o}C for approximately 1 month before development with Kodak D-19.

Preparation of Cell Suspensions from Rodent Lung and Separation of Cell Types by Centrifugal Elutriation.

The cell types of most interest for lung carcinogenesis are the bronchiolar epithelial cells and the type II alveolar cells. This is because most lung tumors in both man and experimental animals seem to arise from these cell types, appearing as bronchogenic carcinomas or bronchioloalveolar adenomas or adenocarcinomas. Therefore, in these studies, we have focused on the metabolic activity in preparations enriched in type II cells or bronchiolar epithelial cells.

Methods for enzymatic dispersal of rodent lung have been described by several groups of workers (Devereux and Fouts, 1981; Jones et al., 1982). In general, the lung is lavaged to remove some of the alveolar macrophages, and then inflated with a solution of proteolytic enzymes in order to disrupt intercellular attachments.

After evaluation of a number of enzymes and enzyme mixtures including Protease I (Sigma), collagenase (Worthington), thermolysin (Sigma), trypsin (Sigma) and hyaluronidase (Sigma), we have found that, for our purposes, a mixture of trypsin and hyaluronidase provides a good yield of type II and bronchiolar epithelial cells. The procedure, described below, is similar for rats, mice, or hamsters, except for the volumes used to lavage the lung.

The animals are anesthetized with sodium pentobarbital (Western Medical Supply, Los Angeles, CA) to which is added sodium heparin (100 units/gm b.w.). The animals are killed by bleeding from the femoral arteries. After opening the thoracic cavity, the lung vasculature is perfused via the pulmonary artery with heparinized Krebs-Ringer bicarbonate saline. A cannula is tied into the trachea, and the lungs lavaged 5 times with warm HEPES-buffered balanced saline solution (HPBS). Finally, the lungs are inflated to capacity with a solution containing 0.05% trypsin, 100 units/mL of hyaluronidase, and 0.05% DNAase I in Ca^{++} and Mg^{++}-free HPBS. The trachea is ligated, and the lungs removed en bloc and incubated for 30 min at 37°C in HPBS. At this time, the lung lobes are removed, minced and stirred with soybean trypsin inhibitor (1 mg/mL, Sigma) in the cold for an additional 30 min. Freed cells are collected by filtration through 40 um nylon mesh and centrifugation. DNAase I is present at all times at a concentration of 0.05%.

Fractionation of the cell suspension by centrifugal elutriation (CE) to obtain preparations enriched in type II or Clara cells was accomplished using the Beckman JE-6 rotor and J6M centrifuge. For loading into the rotor the cells were suspended at approximately 10^7/mL in buffer consisting of 67% HPBS and 33% Ham's F12K (Kaighn, 1973), 0.5% bovine serum albumin (Sigma, Cohn fraction V), and 0.05% DNAase I. This same buffer was used for elutriation. The conditions for elutriation (Table 3) were developed from theoretical considerations and experimental results in our laboratory and others. The initial conditions for loading cells into the rotor were such that erythrocytes and cell debris did not remain in the rotor chamber. Stepwise increase of the flow rate yielded fractions enriched in cells of a particular size range. Since type II and Clara cells are quite different in size, these cells can be readily separated.

Table 3

Typical Separation of Rat Lung Cells by Centrifugal Elutriation[a]

Fraction Number	Flow, mL/Min	Cell Number	% Type II Cells	% Clara Cells
1	7.0	2.4×10^7	18	0
2	9.5	8.4	41	0.6
3	11.0	2.0	47	2.0
4	12.5	1.5	46	4.5
5	14.0	1.3	39	4.9
6	16.0	1.3	37	8.6
7	19.0	0.9	27	5.6
8	32.0	1.0	19	11.8
9	32.0	1.1	11	16.3

[a]Centrifuge speed was 1500 rpm for all fractions except for #9, for which it was zero. Fraction volumes were 150 mL for #1-7 and 100 mL for #8 and #9.

The separated cells were collected by centrifugation, and smears were prepared and stained as described by Dewvereux and Fouts (1981) for identification and enumeration of cell types. The cell pellets were frozen at -70°C until analysis for ^{3}H-BaP metabolizing activity which was done the day following cell separation.

^{3}H-BaP Metabolism

Metabolism of ^{3}H-BaP by cell homogenates was done essentially as previously described (Rasmussen and Wang, 1974; Rasmussen, 1983). Briefly, the cells were homogenized in a minimum volume of TKM-sucrose (0.05 M Tris, 0.025 M KCl, 0.005 M $MgCl_2$, 0.25 M sucrose, pH 7.4) and aliquots containing 0.2-0.5 mg protein were incubated with ^{3}H-BaP (10 uCi/mL, 17.4 Ci/mmole) (Amersham Corp.,) and 1.2 mM NADPH for 30 min. at 37°C in a total volume of 2.0 mL TKM-sucrose. Protein was measured by the Lowry method (Lowry et al., 1951). The reaction was stopped by addition of 2 volumes of ethyl acetate and chilling in ice. Metabolites and unchanged ^{3}H-BaP were extracted with ethyl acetate and the solvent evaporated under dry N_2. The residue was dissolved in a small volume of acetone or methanol and analyzed by either thin layer (TLC) as described previously (Rasmussen and Wang, 1974) or by high performance liquid chromatography (HPLC). The latter analysis used an Altex Ultrasphere ODS column and a concave solvent gradient of 60%-85% methanol in water with a flow rate of 1 ml/min and a rate of change of concentration of approximately 0.5%/min. Metabolites were identified using authentic derivatives of BaP obtained from the National Cancer Institute as chromatography standards. Radioactivity was measured using a Tracor scintillation spectrometer.

Treatment of Hamsters with Diethylnitrosamine (DEN)

Male Syrian golden hamsters were given DEN by i.p. injection of solutions of the chemical in 0.9% NaCl, as appropriate to the experiments described below. The animals weighed 90.0 + 2.5 g at the start of an experiment. Unused DEN was destroyed by treatment with alkali before disposal as contaminated waste.

Isolation of DNA and Analysis for Ethylated Guanine

DNA was isolated by a modified phenol extraction procedure (Swann and Magee, 1968) and hydrolyzed at neutral pH (100°C, 60 min) to preferentially release 7-EtG. The partially apurinic DNA was then precipitated with 1.0 N HCl and subjected to mild acid hydrolysis (0.1 N HCl, 70°C, 40 min) to release O^6-EtG. The hydrolysates were analyzed for the presence of ethylated bases by strong cation exchange HPLC (Partisil-10SCX, 25 cm x 0.45 cm, Whatman, Clifton, NJ). 7-EtG and O^6-EtG were identified and quantified by fluorescence spectroscopy using authentic compounds as reference standards (Becker, 1982).

Analysis of Cell Proliferation in DEN-Treated Hamsters

One hour prior to sacrifice, the hamsters were given 100 uCi of ^{3}H-dThd by i.p. injection. Tissue samples from lung, trachea, and liver were fixed in Karnovsky's solution, embedded in Araldite, and 2 um sections cut. Autoradiographs were prepared by dipping the sections in Kodak NTB-2, as described above for rat lung sections.

EXPERIMENTAL RESULTS AND DISCUSSION

Cell Proliferation in Lungs of Rats Treated with MCA

Lungs of rats treated at the THRU five times with 1.0 mg MCA, and labeled as described with ^{3}H-dThd before killing were analyzed for radioactivity. Table 4 summarizes the results expressed as ^{3}H-dpm per mg lung dry weight. There were no significant differences among any of the treatment groups, nor was there any change in labeling with the age of the rats over the period of the experiment.

Table 4

Specific Radioactivity of Rat Lung Tissue Labeled In Vivo with ^{3}H-dThd[a]

SAC. DATE	SHELF CONT.	GEL-SALINE	MCA, 5 x 1.0 MG
5-9-83	Not Sampled	597 ± 113[c]	662 ± 208[c]
6-13-83	525 ± 61[c]	534 ± 49[b]	494 ± 123[c]
7-18-83	Not Sampled	532 ± 64[c]	560 ± 39[c]
8-22-83	683 ± 160[c]	466 ± 73[d]	461 ± 71[d]
12-5-83	623 ± 109[c]	466 ± 24[c]	512 ± 86[c]

[a] Values are ^{3}H-dpm per mg dry weight ± S.D. and are based on samples from each lung lobe of every rat, i.e., 5 samples per rat.
[b] Value based on 1 rat.
[c] Values based on 2 rats.
[d] Values based on 4 rats.

The dose of MCA chosen (5 x 1 mg) was expected to produce lung tumors in about 50% of the rats. Among the 21 MCA-treated rats examined, 7 were found with squamous cell carcinoma (SCC) of the lung. (Table 5) Two additional rats had marked focal hyperplasia of the epithelial cells of the terminal bronchioles, which may have been pretumorous lesions (Shabad and Pylev, 1970). In these rats, the bronchiolar epithelial cells appeared to invade the alveolar spaces, and alveoli became lined with ciliated and non-ciliated epithelial cells which closely resembled the cells of the normal terminal bronchioles. No other significant lesions were seen. Since 9 of the 21 rats had tumors or pretumorous lesions, the expected tumor yield was apparently obtained.

Table 5

Lung Lesions and Tumors Found in Rats Treated 5 Times with MCA

SAC.DATE	SHELF CONT.	GEL-SALINE	5 x 1.0 MG MCA
5-9-83	No Samples	N.L./ 2 Rats	1 Rat TBH; 1 N.L.
6-13-83	N.L./ 2 Rats	N.L./ 2 Rats	2 Rats SCC; 3 N.L.
7-18-83	No Samples	N.L./ 2 Rats	1 Rat SCC; 1 N.L.
8-22-83	N.L./ 2 Rats	N.L./ 2 Rats	2 SCC; 1 TBH; 1 N.L.
12-5-83	No Samples	N.L./ 2 Rats	1 SCC; 1 N.L.
2-13-84	N.L./ 6 Rats	N.L./ 6 Rats	1 SCC; 5 N.L.

N.L. = No Significant Lesions; TBH = Terminal Bronchiolar Epithelial Hyperplasia; SCC = Squamous Cell Carcinoma.

One aim of this study was to determine whether exposure to a potentially carcinogenic dose of a known carcinogen would produce effects on lung tissue, either through cytotoxicity or other mechanisms, that would be detectable as increased ^{3}H-dThd incorporation in lung tissue. The results indicate that, for MCA, this is not the case. Even when tumors were present in the lung, and proliferating cells were evident by autoradiography, the overall uptake of ^{3}H-dThd could not be distinguished from control values.

Cell Proliferation in Rat Lung after Treatment with MCA and/or Chrysotile Asbestos

The dose of MCA chosen for this experiment was expected to produce tumors in less than 10% of the rats (Rasmussen, 1981). Therefore, if asbestos had a tumor-enhancing effect, it should have been apparent. The dose of asbestos (5 x 1.0 mg) was chosen so as to be tolerated by the rats without mortality. It should be noted that the asbestos preparation method excluded the longer asbestos fibers which have been implicated as causing lung cancer in other experimental systems (Stanton et al., 1981). The aim of this study was to determine the effect of shorter fibers which could be inhaled into the deep lung.

Measurement of radioactivity in lungs of rats given ^{3}H-dThd prior to sacrifice did not reveal any significant differences among the treatment groups (Table 6). The values of ^{3}H-dpm/mg dry weight were similar in all groups throughout the course of the experiment.

Table 6

Specific Radioactivity of Lung Tissue from Rats Treated with MCA and/or Asbestos and Labeled In Vivo with ^{3}H-dThd[a]

SAC. DATE / ASBESTOS	GEL-SALINE	ASBESTOS	MCA	MCA +
6-20-83[b]	770 ± 133	668 ± 75	873 ± 193	583 ± 252
7-25-83	542 ± 65	685 ± 56	742 ± 75	684 ± 147
9-5-83	552 ± 53	582 ± 69	503 ± 56	461 ± 99
10-17-83	554 ± 95	577 ± 47	553 ± 102	672 ± 131
12-12-83	529 ± 62	557 ± 54	573 ± 85	780 ± 180

[a] Values are as in Table 4.
[b] Values based on 2 rats; All others based on 5 rats.

Examination of lung sections showed a few small foci of hyperplasia of the terminal bronchiolar epithelium of rats treated with both MCA and asbestos, and in 2 rats these changes may have been pretumorous. No definite SCC were found in any rats.

In an experiment conducted at UCI, the dose of MCA was increased to 5 x 1.0 mg while using the same dose of asbestos as in the experiment conducted at the THRU. Lung slices were incubated with ^{3}H-dThd in vitro as described previously (Rasmussen, 1981). Autoradiographs were prepared and the fraction of labeled alveolar cells was determined by direct count. The results (Table 7) indicated that, in general, the labeling index in lungs of rats treated with MCA or MCA plus asbestos was slightly higher than controls or rats treated with asbestos only. However, the only statistically significant values were those found in lungs of rats killed 24 hours after the last intratracheal treatment.

Table 7

^{3}H-dThd Labeling Index in Lungs of Rats Treated 5 Times with MCA[a]

DAYS POST-TREATMENT	RATS/GROUP	CONTROLS	ASBESTOS	MCA	MCA + ASB.
1	2	10.2 ± 6.0	11.0 ± 2.6	22 ± 9.5[b]	19 ± 4.7[c]
7	2	14.5 ± 7.2	4.7 ± 2.4	8.6 ± 4.8	10 ± 7.0
30	2	1.6 ± 1.1	1.6 ± 0.7	3.1 ± 2.2	2.8 ± 4.4
60	2	4.0 ± 1.0	2.1 ± 1.9	8.5 ± 3.4	6.0 ± 1.9
90	3	3.4 ± 1.6	3.6 ± 1.6	5.3 ± 2.2	4.1 ± 2.1
120	3	2.4 ± 1.9	3.9 ± 2.7	4.7 ± 3.2	4.7 ± 2.2
150	3	4.0 ± 2.9	4.4 ± 2.1	5.7 ± 2.9	5.0 ± 2.5

[a] Values are the average number of labeled cells per 1000 cells counted, ± S.D., based on a total count of 5000 cells per rat.
[b] Significantly greater than control, p 0.01, t = 3.26.
[c] Significantly greater than control, p 0.01, t = 2.95.

The results of these experiments indicated that no lasting overall stimulation of cell proliferation in the rat lung was produced by treatment expected to lead to tumor formation.

Separation of Lung Cell Types by CE

Table 3 shows the results of a typical experiment in which rat lung cells were separated by CE. Type II cells are found distributed among all fractions, but are most concentrated relative to Clara cells in fractions 2 and 3. Clara cells and other epithelial cells are found mostly in the later fractions, especially 8 and 9. A problem in separation of Clara cells from other cell types is that the bronchiolar epithelial cells are not released from the lung as single cells, but mostly as clumps of 3 or more cells containing both ciliated and non-ciliated cell types. This has also been observed by others (Jones et al., 1982). Thus, the "Clara cell" fractions really consist of mixed Clara and other epithelial cells. On the other hand, the fractions enriched in type II cells are almost entirely monodisperse and contain few Clara or other cell types. The degree of separation obtained to date has provided some data on the enzymatic activity of the different cell types, but further work is necessary to improve the purity of the preparations.

Preliminary experiments with the mouse have indicated that conditions for separation of lung cell types are similar to those used for the rat. Table 8 shows results of a typical experiment. The distribution of type II and Clara cells was very similar to that found with rat lung cells.

Metabolism of ^{3}H-BaP by Isolated Lung Cells

Experiments with untreated rats and mice indicated that lung cells normally have very low levels of the P-450 enzymes involved in metabolism of compounds such as BaP. Therefore, in the work presented here, the animals were treated 48 hours before sacrifice with an inducer of P-450 enzymes, beta-naphthoflavone (BNF) at a dose of 80 mg/kg b.w.. BNF was given i.p. as a peanut oil solution.

Table 8

Separation of Mouse Lung Cells By Centrifugal Elutriation[a]

FRACTION	FLOW, ML/MIN	CELL YIELD	% TYPE II	% CLARA
1	7.0	1×10^8	N.D.	0
2	9.5	3.8×10^7	36	0
3	11.0	8.8×10^6	52	0
4	12.5	4.9×10^6	41	0.75
5	14.0	6.5×10^6	23	2.8
6	16.0	5.4×10^6	14	4.2
7	19.0	3.3×10^6	11	12
8	32.0	6.0×10^6	6.6	20
9	32.0	8.5×10^6	4.5	22

[a] Rotor speed was 1500 rpm for all fractions except for #9 for which it was zero. Fraction volumes were 150 ml for fractions 1-7 and 100 ml for fractionsa 8 and 9. N.D. = not determined.

Enzymatic activity toward ^{3}H-BaP was present in all cell fractions but was highest in the fractions enriched in Clara cells. In the case of the rat, there was also a difference in the relative yields of metabolites of ^{3}H-BaP produced by type II-enriched and Clara cell-enriched fractions. This was shown most clearly by HPLC analysis of ^{3}H-BaP metabolism by rat lung cells. Table 9 shows the results of this experiment. The Clara cell-enriched fractions produced relatively more of the dihydrodihydroxy (diol) derivatives of BaP compared to the type II-enriched fractions. Since the ultimate carcinogenic form of BaP is likely produced by metabolism of the 7,8-diol to the 7,8-diol-9,10-oxide (Sims et al., 1974; Thakker et al, 1976), these findings suggest that Clara, or other bronchiolar epithelial cells may experience greater risk of neoplastic transformation than type II cells. This hypothesis is supported by the fact that hydrocarbons such as BaP induce lung carcinomas that appear to arise from bronchiolar epithelial cells.

Metabolism of ^{3}H-BaP by isolated mouse lung cells was most active in the Clara cell-enriched preparations (Table 10). However, in contrast to the rat, there was no difference between Clara cells and type II cells in the relative amounts of metabolites formed. The percentage yields of the individual metabolites differed slightly from the rat, but most closely resembled the profile seen with the rat Clara cell-enriched fractions.

These experiments have demonstrated that lung cells can be isolated from rats and mice, and that preparation enriched in specific lung cell types can be obtained and their metabolic activity measured. Future studies will focus on further purification of type II and Clara cells, and comparative studies among the rat, mouse, and hamster of their enzymatic activities.

Accumulation of 7-EtG and O^6-EtG During Chronic Exposure to DEN

Hamsters were given 20 mg DEN/kg b.w. by s.c. injection twice weekly. At intervals up to 8 weeks, sample animals were killed and lung, liver and kidney were frozen at -80°C until isolation of DNA. At each sacrifice time, 2 groups of hamsters were killed, one at 3 hours and one at 24 hours after the last DEN treatment.

Table 9

HPLC Analysis of ^{3}H-BaP Metabolites Produced by Isolated Rat Lung Cells

METABOLITE	TIME,MIN	TYPE II (66%)	CLARA (13%)
Unknown	2.8	3 (0.3%)[a]	26 (1.8%)
9,10-Diol	6.3	63 (6.9%)	250 (17.2%)
Unknown	9.8	17 (1.8%)	38 (2.6%)
4,5-Diol	17.0	81 (8.9%)	222 (15.3%)
7,8-Diol	19.9	67 (7.3%)	163 (11.2%)
Quinones	25-32	180 (18.7%)	343 (23.6%)
9-Hydroxy	34.2	141 (15.4%)	123 (8.5%)
3-Hydroxy	35.5	364 (39.8%)	287 (19.8%)

[a] Values are picomoles of metabolites produced per mg protein in a 30 min incubation period, and are the average of duplicate samples. Percentages in parentheses are the fraction of total metabolite yield appearing as that metabolite. Time values are the time of elution of the metabolites from the HPLC column.

Table 10

^{3}H-BaP Metabolism by Isolated Mouse Lung Cells[a]

FRACTION #	9,10-DIOL	7,8-DIOL	4,5-DIOL	BAP-OH	QUINONES
2	16	34	17	55	89
3	21	43	24	79	78
4	12	38	30	81	96
5	13	42	23	100	23
6	14	41	25	119	74
7	19	48	40	89	250
8	67	136	93	243	307
9	148	228	132	434	326

[a] Values are picomoles of metabolite per mg protein produced during a 30 min incubation period, and are the average of duplicate samples. The percentage of type II or Clara cells in each fraction are given in Table 8. Analysis was by TLC.

Figure 1 shows the accumulation of O^{6}-EtG and 7-EtG in liver DNA of hamsters receiving up to 16 s.c. injections of DEN. No detectable ethylation was found in the liver of any control hamster. O^{6}-EtG was detected at 3 and 24 hr after a single DEN treatment and the concentration of this adduct increased during chronic exposure, reaching a maximum at week 8. In contrast, 7-EtG did not accumulate during the chronic exposure.

In lung DNA (Figure 2), O^{6}-EtG concentrations increased after 2 weeks of treatment to twice that seen after a single injection. The concentrations of O^{6}-EtG remained constant between 2 and 4 weeks but dropped to single injection levels at 8 weeks. 7-EtG concentrations varied during the experiment but did not show any tendency toward accumulation. No ethylation was detected in control animals.

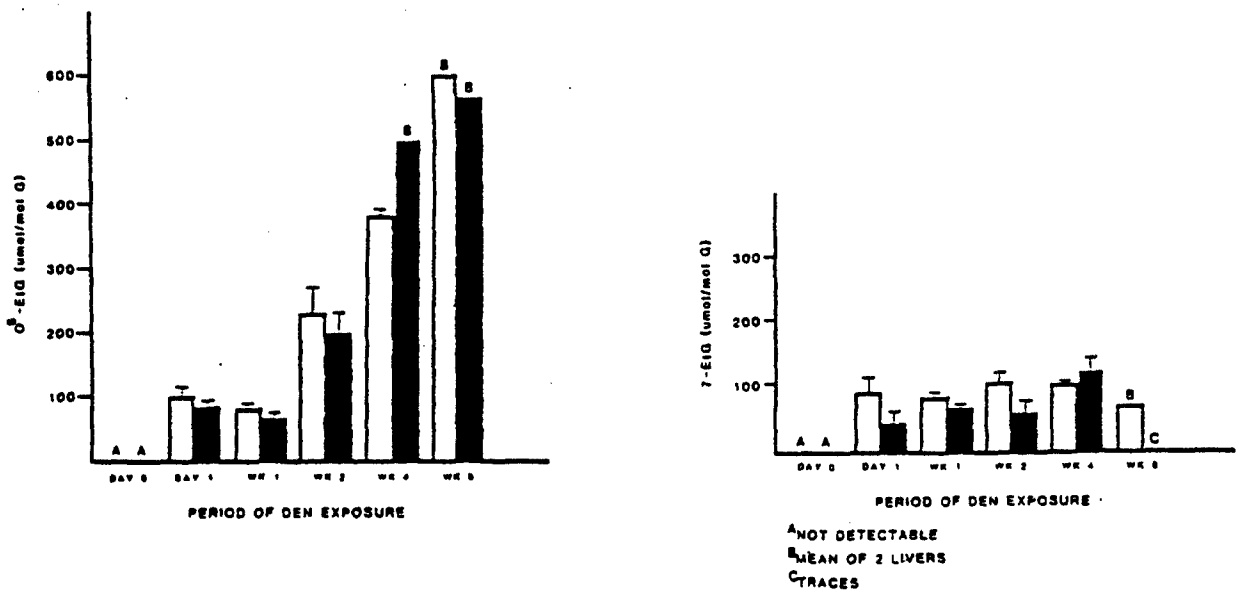

Figure 1. Levels of ethylguanines in liver DNA of hamsters given DEN, 20 mg/kg b.w., twice weekly by s.c injection for up to 8 weeks. Open bars, animals killed at 3 hr after the last injection; Solid bars, animals killed at 24 hr after the last injection. Values are the mean of determinations on 3 individual livers; error bars are S.E.

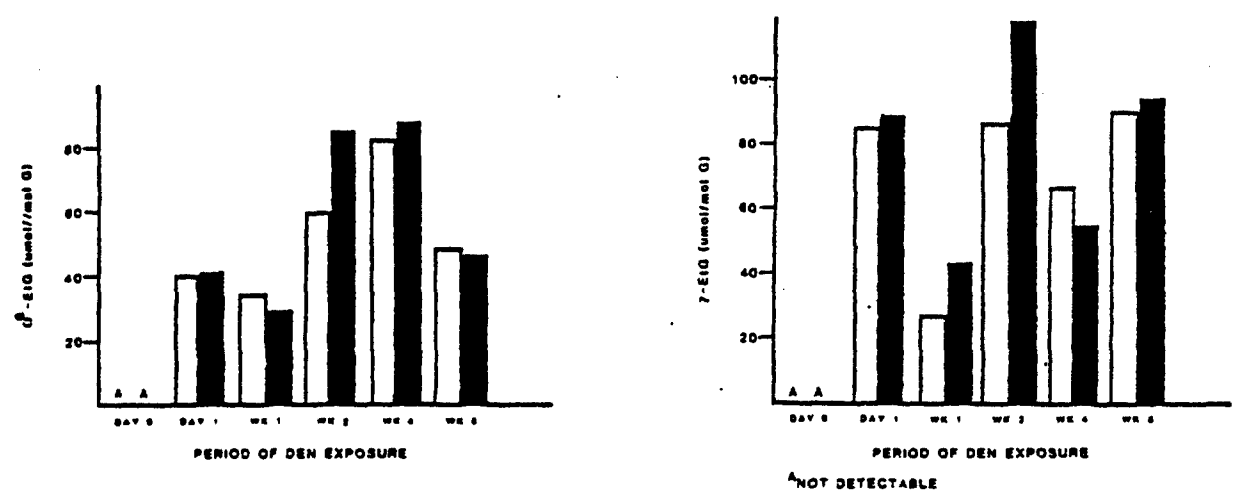

Figure 2. Levels of ethylguanines in lung DNA of hamsters treated as in Figure 1. Symbols are as in Figure 1. Values are the average of values obtained from 2 groups of hamsters with 5 pooled lungs per group. Individual determinations for each time point were within 15% of each other.

Ethylation was not detected in the kidney at early times of the experiment, but traces of O^6-EtG were found at 2, 4, and 8 weeks, and traces of 7-EtG were found at 1, 2, 4, and 8 weeks. The kidney is not a target for DEN carcinogenesis.

Formation and Removal of O^6-EtG after DEN Challenge

Hamsters were given DEN, 20 mg/kg b.w., or 0.9% NaCl by s.c. injection twice weekly for 4 weeks. At 24 hr after the last DEN treatment, the animals were challenged with a single i.p. injection of DEN, 150 mg/kg b.w. The hamsters were killed at 3, 12, 24, and 48 hr after the i.p. injection. The DNA was isolated and examined as in the chronic studies.

Figure 3 shows the concentrations of O^6-EtG present in hamster liver and lung DNA at various times after the i.p. challenge dose. The highest levels of O^6-EtG were detected in both lung and liver DNA at 12 hr after the challenge dose. DEN pretreatment did not appear to inhibit or enhance the rate of removal of O^6-EtG from liver or lung DNA. DEN pretreatment did not change the maximum O^6-EtG levels found at 12 hr after the challenge dose. O^6-EtG concentration in liver DNA at 3 hr after the challenge dose was higher in the pretreated hamsters compared to the saline pretreated controls. It is important to note that after 4 weeks of DEN treatment, 502.6 umol of O^6-EtG per mole of guanine was found in the liver 24 hr after the last s.c. injection. Therefore residual O^6-EtG from DEN pretreatment contributed to the higher level of O^6-EtG in the liver DNA of the pretreated hamsters found at 3 hr after the challenge dose. The levels of O^6-EtG were lower in the lung compared to the liver DNA at all time points studied. The rate of removal of O^6-EtG from liver DNA was 2-3 times that of lung DNA. O^6-EtG was not detected in the kidney DNA at any time following the DEN challenge dose.

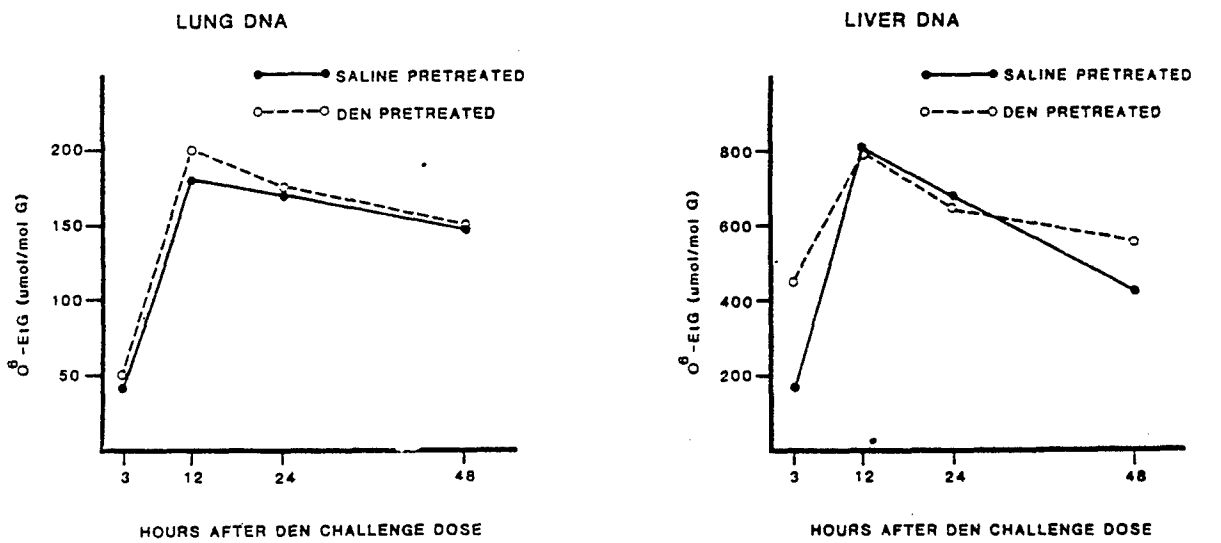

Figure 3. Levels of ethylguanines in lung and liver DNA of hamsters pretreated with either DEN (20 mg/kg b.w.) or 0.9% NaCl given s.c. twice weekly for 4 weeks, and then challenged with a single large dose of DEN (150 mg/kg b.w.) given i.p. 24 hr after the last s.c. injection.

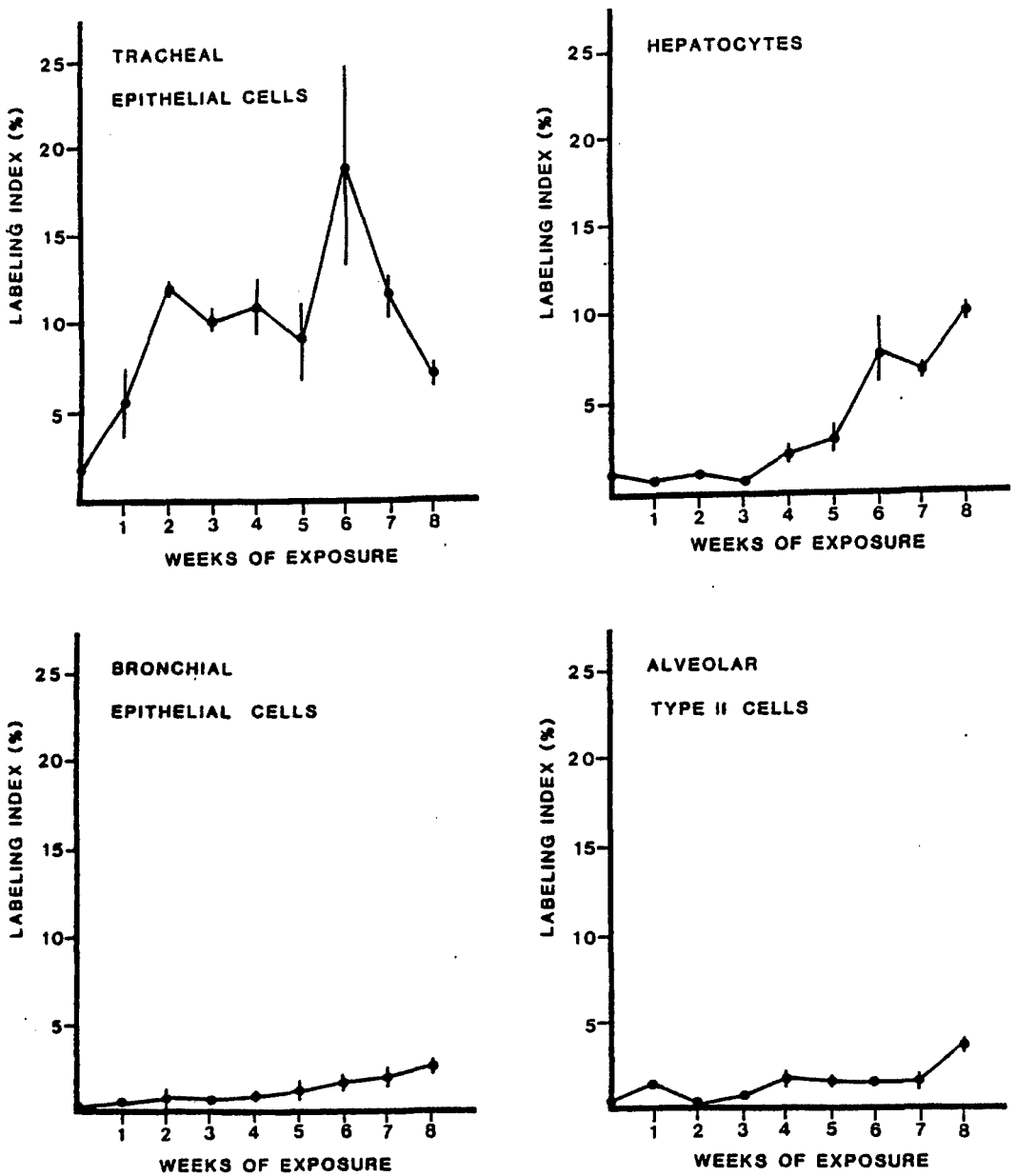

Figure 4. Labeling index in liver and lung of hamsters given DEN, 20 mg/kg b.w., twice weekly by s.c injection for up to 8 weeks. Error bars are S.E.

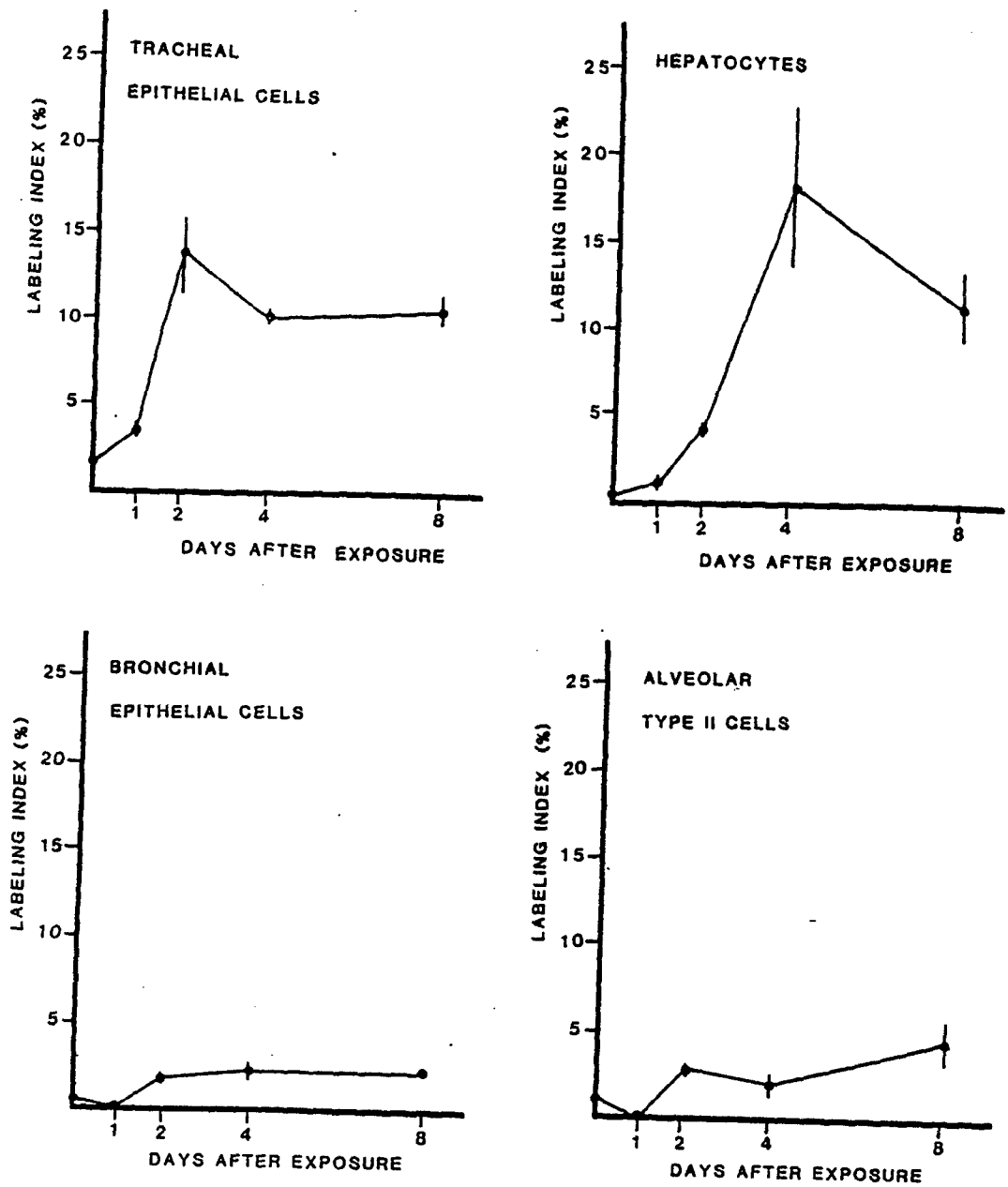

Figure 5. Labeling index in liver and lung of hamsters given a single i.p. injection of DEN at a dose of 150 mg/kg b.w. Error bars are S.E.

^{3}H-dThd Incorporation into Cellular DNA During Chronic and Acute DEN Exposure

Hamsters in the chronic study were given DEN, 20 mg/kg b.w., twice weekly by s.c. injection for up to 8 weeks, and hamsters in the acute study were given DEN, 150 mg/kg b.w. by a single i.p. injection. Control hamsters received 0.9% NaCl only. The incorporation of ^{3}H-dThd into cellular DNA was measured as the labeling index which is the ratio of the number of labeled nuclei to the total number of nuclei.

In the chronic study, the labeling index in tracheal epithelial cells increased rapidly to a maximum level of labeling of 19% at week 6 (Figure 4). In contrast the effect of DEN on ^{3}H-dThd incorporation into type II alveolar cells was much less pronounced. The labeling index increased slowly during the course of the chronic treatment. A small progressive increase was also found in the bronchial epithelial cells. In hepatocytes, DEN had little effect on ^{3}H-dThd incorporation during the first 4 weeks of exposure, but caused a sharp increase in the second 4 weeks. Control hamsters killed at various times during the exposure period did not differ significantly in ^{3}H-dThd incorporation.

In the respiratory tract, the tracheal epithelial cells were selectively affected by an acute exposure to DEN (Figure 5). Liver necrosis was seen 4 days after the acute exposure, and coincided with a large increase in the labeling index of hepatocytes. Bronchiolar epithelial cells and alveolar type II cells also showed a slight increase in the labeling index suggesting that these cells may be sensitive to the toxic effects of DEN.

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* U.S. GOVERNMENT PRINTING OFFICE: 1984 - 559-006/10215